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The Antidepressant Sertraline Provides a Promising Therapeutic Option for Neurotropic Cryptococcal Infections

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Therapeutic treatment for systemic mycoses is severely hampered by the extremely limited number of antifungals. The difficulty of treatment of fungal infections in the central nervous system is further compounded by the poor central nervous system (CNS) penetration of most antifungals due to the blood-brain barrier. Only a few fungistatic azole drugs, such as fluconazole, show reasonable CNS penetration. Here we demonstrate that sertraline (Zoloft), the most frequently prescribed antidepressant, displays potent antifungal activity against *Cryptococcus neoformans*, the major causative agent of fungal meningitis. In *in vitro* assays, this neurotropic drug is fungicidal to all natural *Cryptococcus* isolates tested at clinically relevant concentrations. Furthermore, sertraline interacts synergistically or additively with fluconazole against *Cryptococcus*. Importantly, consistent with our *in vitro* observations, sertraline used alone reduces the brain fungal burden at an efficacy comparable to that of fluconazole in a murine model of systemic cryptococcosis. It works synergistically with fluconazole in reducing the fungal burden in brain, kidney, and spleen. In contrast to its potency against *Cryptococcus*, sertraline is less effective against strains of *Candida* species and its interactions with fluconazole against *Candida* strains are often antagonistic. Therefore, our data suggest the unique application of sertraline against cryptococcosis. To understand the antifungal mechanisms of sertraline, we screened a whole-genome deletion collection of *Saccharomyces cerevisiae* for altered sertraline susceptibility. Gene ontology analyses of selected mutations suggest that sertraline perturbs translation. *In vitro* translation assays using fungal cell extracts show that sertraline inhibits protein synthesis. Taken together, our findings indicate the potential of adopting this antidepressant in treating cryptococcal meningitis.

Systemic fungal infections have increased dramatically worldwide during the past 3 decades due to the growing immunocompromised population, resulting from organ and tissue transplantation, cancer therapy, steroid therapy, and HIV infection (2, 18). *Cryptococcus neoformans*, one of the major opportunistic fungal pathogens, can cause systemic infections that commonly involve the central nervous system (CNS) (5, 18). Cryptococcal meningitis is always fatal without treatment. Approximately 25% to 30% of AIDS patients worldwide (>0.6 million) die each year as a consequence of this disease (24). The serious threat caused by *Cryptococcus* is further exacerbated by the limitations of the current antifungal therapy for treatment of cryptococcosis.

The available repertoire of antifungals against *Cryptococcus*, as well as other fungal pathogens, is extremely limited in number. The newly developed echinocandins that target fungal β-1,3-glucan synthase are promising in treating infections caused by *Aspergillus* and *Candida* species, but they are ineffective against *Cryptococcus* species (17). The standard treatment for AIDS-related cryptococcal meningitis still relies on a 2-week induction treatment with amphotericin B (or liposomal amphotericin B), followed by consolidation and lifelong maintenance therapy with azole drugs, especially fluconazole (FLC) (26). Amphotericin B was discovered in the 1950s, and its use is limited to the initial treatment due to its toxicity (11, 26). Fluconazole is well tolerated by patients and shows good penetration into the CNS (3). However, fluconazole is fungistatic rather than fungicidal (9). Because fungal infections of the brain have poor prognoses (5) and because negative brain cultures are not always achievable with current anticryptococcosis therapy (4), it is critical to develop antifungals that can penetrate the CNS and clear the brain fungal burden.

Given the challenges of *de novo* antifungal drug development, identification of existing clinical compounds that exert fungicidal activity represents a reasonable approach to improve current antifungal treatment. During our previous screen of the Johns Hopkins Clinical Compound Library (40), we identified a modest growth-inhibitory effect of sertraline (SRT) against the filamentous fungus *Aspergillus nidulans*. Potential antifungal activity of this antidepressant was first observed in a clinical setting: three patients with premenstrual dysphoric disorder (PMDD) and recurrent vulvovaginal candidiasis (VVC) were treated with sertraline for their PMDD. Their clinical symptoms of VVC disappeared during the sertraline therapy but recurred after treatment was stopped (13). Follow-up *in vitro* studies demonstrated that sertraline is fungicidal against *Aspergillus* and *Candida*, with MICs ranging from 7 to >256 μg/ml (12, 13). Because the MICs of sertraline against these *Aspergillus* and *Candida* strains are much higher than the serum concentrations of sertraline (55 to 250 ng/ml) achievable in standard therapeutic regimens (34), the potential clinical value of sertraline in treating systemic infections caused by these fungi was considered low (37).

The data presented in this report demonstrate that sertraline is particularly potent against *Cryptococcus*, with its fungicidal concentrations falling below 10 μg/ml. Furthermore, previous pharmacokinetics studies of sertraline in rats and dogs indicate that sertraline concentrations in cerebrospinal fluid, brain, and some

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other organs are 20- to 40-fold higher than its serum concentration (31). Such levels could meet or exceed the MICs of sertraline against *Cryptococcus* isolates. In addition, abundant clinical data support the safety of long-term use of this antidepressant (6, 28, 29). Given its psychotropic nature and its potent fungicidal activity against *Cryptococcus*, we hypothesized that sertraline could be uniquely suitable in treating systemic cryptococcosis.

The mechanism of action of sertraline in the mammalian nervous system has been well characterized: it blocks the 5-hydroxytryptamine (5-HT) transporter and inhibits the reuptake of 5-HT into the presynaptic cell (1). However, there is no conserved homolog of the 5-HT transporter in fungi. Thus, the underlying mechanisms responsible for the antifungal activity of sertraline remain mysterious. One report attributed the antifungal activity of sertraline to a nonspecific cytotoxicity due to its lipophilicity (37). However, findings of two recent studies suggested membrane organization and vesicle-mediated transport as its antifungal targets (27, 30). Sertraline was also shown to possess antitumor activity (15, 16, 32). The antitumor activity was attributed to the inhibition of the initiation of protein synthesis through its effects on the mTOR pathway (15). It is not clear if translation is also a target of sertraline in fungal cells, as homologs of certain relevant factors important for translational regulation in mammals do not exist in fungi. In addition to antifungal and antitumor activities, sertraline has been shown to exert antiviral activity (14), antibacterial activity (19, 20), antiparasite activity (23), and spermicidal activity (10). The antiproliferation activity of sertraline against evolutionarily diverse organisms indicates its interference with some fundamental processes. Based on our genetic screen and *in vitro* translation assays using fungal cell extracts, we demonstrate here that sertraline indeed inhibits protein synthesis in fungi. Thus, together with previous studies, our results indicate that the broad antiproliferation activity of sertraline might be attributable to its interference with translation.

MATERIALS AND METHODS

Strains and media. The strains used in this study are listed in Table S1 in the supplemental material. All yeast strains were maintained on yeast extract-peptone-dextrose (YPD) medium. Drug disk diffusion and microdilution assays were performed using RPMI 1640 medium buffered with MOPS (morpholinepropanesulfonic acid) according to the standard protocol of CLSI.

Compounds and animals. Sertraline hydrochloride was dissolved in dimethyl sulfoxide (DMSO) at a stock concentration of 20 mg/ml. The drug was diluted with water or with appropriate media to the indicated working concentrations in the *in vitro* studies. For animal studies, sertraline was dissolved in a 0.9 volume of water first, and then a 0.1 volume of 10× phosphate-buffered saline (PBS) stock solution was added. Fluconazole was dissolved at a stock concentration of 2 mg/ml in water and was diluted by water or appropriate media in the *in vitro* studies. Fluconazole was dissolved in 1× PBS buffer at 3 mg/ml for the animal studies. Female A/J mice (10 to 12 weeks old) were purchased from Charles River (Wilmington, MA). Drug solutions for animal studies were freshly prepared before daily injection.

***In vitro* study of antifungal activity.** The drug disk diffusion assay was independently performed four times as previously described (40). The microdilution assay was performed according to the CLSI standard except that cells were incubated at 37°C to mimic host conditions. The initial inocula of fungal cells ranged from 1,500 to 2,000 cells/ml. Drugs were added by calculating the desired dose as indicated. The MICs were defined by comparing the growth of the treated samples after 48 h of incubation for *Cryptococcus* strains or 24 h of incubation for *Candida* strains versus the no-drug control via measuring the absorbance at 600 nm. The minimum fungicidal concentrations (MFCs) were defined as those at which at least 99% of cells were killed compared to the original inocula. The number of viable cells was determined by plating the suspension on drug-free solid media and then measuring CFU.

The interaction between sertraline (SRT) and fluconazole (FLC) was quantified by the fractional fungicidal concentration index (FFCI) as follows: FFCI = [FLC]/MFC_{FLC} + [PMB]/MFC_{SRT}, where PMB represents polymyxin B, MFC_{FLC} and MFC_{SRT} represent the concentrations of fluconazole and sertraline used alone, respectively, and [FLC] and [SRT] represent the concentrations at which fluconazole and sertraline in combination killed at least 99% of cells compared to the original inocula. When MFC was not achieved (">" was used in these cases), the concentration 2× that of the highest concentration tested was used for the FFCI calculation. For the interaction of sertraline and fluconazole against *Candida* strains, the status of cell proliferation was measured by the absorbance at 600 nm and is quantitatively displayed using gradations of color.

***In vivo* model of systemic cryptococcosis.** Mice were assigned to 4 treatment groups: control (PBS), fluconazole, sertraline, and the drug combination. All treatments, including the PBS control, were given intraperitoneally. The mice of the sertraline and the drug combination groups were treated with sertraline at a dose of 15 mg/kg of body weight/day for 7 days before *Cryptococcus* infection. All the mice were intravenously challenged with 1.0×10^4 H99 cells. The sertraline treatment was continued. The fluconazole treatment at the dose of 15 mg/kg/day and treatment of the drug combination group were initiated after 24 h of infection. All the drug treatments continued for 3 consecutive days after infection, and the mice were sacrificed. Brains, kidneys, and spleens of mice were harvested and homogenized. Suspensions were serial diluted (10×), plated onto YPD agar, and incubated for another 2 days such that the colonies became visible in order to calculate CFU. Animals were weighed daily and monitored twice a day for disease progression and potential severe side effects, including weight loss, gait changes, labored breaths, or fur ruffling. The animal experiments were carried out in strict accordance with the recommendations in the *Guide for the Care and Use of Laboratory Animals* of the National Institutes of Health. The protocol was approved by the Texas A&M University Institutional Animal Care and Use Committee (IACUC; animal protocol permit 2011-22).

The *Saccharomyces cerevisiae* gene deletion mutant screen for sertraline-resistant or sertraline-sensitive mutants. In the initial screen, organisms from the genome deletion mutant collection were replicated in 96-well plates on RPMI agar medium and on RPMI agar medium with the addition of sertraline at 4 μg/ml and 6 μg/ml for the selection of sensitive and resistant strains, respectively. After 2 days of incubation, about 300 strains with visually detectable differences in colony growth (representing those that were either more sensitive or more resistant) were selected. These selected strains were streaked onto medium for individual colonies and tested through two additional rounds of experiments to exclude issues of contamination during replication or storage. In total, 89 resistant mutants and 33 sensitive mutants were selected. The mutations in several of the most resistant and sensitive strains were confirmed by PCR.

***In vitro* translation assay.** *Cryptococcus* H99 cells from an overnight culture were inoculated into 2 liters of YPD medium to an optical density at 600 nm (OD_{600}) of 0.05. The culture was incubated at 30°C with shaking for approximately 10 more hours to reach an optical density of 2. At this stage, the cells were collected, washed, and resuspended using the same procedure as previously described for the preparation of yeast cell extract (36). The resuspended cells were dripped directly into liquid nitrogen by using a sterile Pasteur pipette to form small ice beads. Frozen cell beads were transferred into prechilled grinding vials of a Spex SamplePrep 6850 Freezer/Mill and powdered in the liquid nitrogen-filled tub using a setting consisting of 10 min of precooling followed by three 2-min grind cycles with 1 min of recooling between cycles. The powdered cells were transferred to prechilled 50-ml polycarbonate centrifuge tubes, allowed to thaw on ice, and then centrifuged at 4°C for 15 min at 16,000 rpm

TABLE 1 Sertraline, alone or with fluconazole, is potent against diverse *Cryptococcus* isolates

Strain	Serotype (source[a])	Geographic origin	SRT		FLC		SRT–FLC	
			MIC_{90}[b]	MFC[c]	MIC_{90}[b]	MFC[c]	MFCs[c]	FFCI[d]
H99	A (C)	North America	6	10	1	8	2–2	0.45
C45	A (C)	North America	2	6	2	6	1–2	0.50
A7-35-23	A (E)	North America	3	10	4	>64	4–4	0.43
C23	A (C)	North America	5	8	8	64	2–8	0.38
Bt31	A (C)	Africa	4	8	12	32	4–8	0.63
Bt81	A (C)	Africa	5	8	12	>64	2–16	0.38
92BC2-45	A (C)	Europe	6	10	4	>64	6–32	0.85
92BC1-52	A (C)	Europe	3	8	3	64	2–4	0.31
98BC1-86	A (C)	Europe	5	8	2	32	2–16	0.75
A2-102-5	A (E)	North America	5	8	16	32	4–8	0.75
123.96	A (C)	North America	4	8	8	>64	4–16	0.63
Bt50	A (C)	Africa	6	10	16	>64	4–32	0.65
163.99	A (C)	North America	4	6	6	>64	2–32	0.58
UA 1993	A (C)	Europe	5	8	2	6	2–2	0.54
JEC21	D (L)	North America	2	6	1	4	1–2	0.83
3-10	D (E)	North America	3	10	4	>64	2–8	0.26
3-17	D (E)	North America	4	8	2	64	2–4	0.31
93BC2-52	D (C)	Europe	3	8	4	32	1–8	0.38
99BC1-40	D (C)	Europe	4	8	3	32	2–4	0.38
UA 491	D (C)	Europe	4	8	2	8	2–2	0.50
XL1495	AD hybrid (L)	North America	4	8	3	8	2–4	0.75
UM4	AD hybrid (N)	Europe	4	10	6	>64	4–16	0.53
92C	AD hybrid (N)	Europe	4	10	4	>64	6–32	0.85
VPCI 87	*C. gattii* (E)	North America	4	10	6	64	2–8	0.33

[a] (C), clinical strain; (E), environmental strain; (L), laboratory strain; (N), not available.
[b] MIC_{90} (shown in micrograms per milliliter), the lowest drug concentration that resulted in a 90% decrease in absorbance.
[c] MFC (minimal fungicidal concentration) (shown in micrograms per milliliter), the lowest concentration at which at least 99% of cells were killed compared to the original inoculums. When fluconazole was used alone, the MFC was sometimes not achieved in the dose range tested, as indicated by ">."
[d] FFCI, fractional fungicidal concentration index. FFCIs < 0.5, drug effects were synergistic; FFCIs between 0.5 and 1.0, drug effects were additive; FFCIs between 1.0 and 2.0, drug effects were indifferent; FFCIs > 2.0, drug effects were antagonistic (4).

in an SS34 rotor. The supernatant was carefully collected with a sterile Pasteur pipette, avoiding both the pellet and the fatty upper layer, and placed in a fresh 15-ml conical tube that was maintained on ice. Small molecules were removed from the extract by chromatography through Sephadex G-25 superfine columns (36).

Capped, polyadenylated RNA encoding the firefly luciferase was synthesized using T7 RNA polymerase from a plasmid template (pPQ101; previously named pHLuc+NFS4 [33, 36]) linearized with EcoRI (for polyadenylated RNA) or XbaI (for nonpolyadenylated RNA). Uncapped RNA transcripts were also synthesized using the same procedure by omitting the cap analog. The yield of RNA was quantified using ImageQuantTL software and ethidium bromide-stained agarose gels by comparison to standard markers with known masses. Gel images were acquired with a GE Typhoon Trio phosphorimager.

The *in vitro* translation reaction mixtures (10 μl; programmed with 60 ng of template RNA) were incubated at 26°C for 40 min as described previously (36). The reaction mixtures prepared in *Cryptococcus* extract contained 110 mM KOAc, 35 mM HEPES-KOH (pH 7.6), 2.6 mM $Mg(OAc)_2$, and 10 μM each amino acid. Translation reactions were stopped by adding passive lysis buffer (Promega) for a final 1× concentration. Firefly luciferase activity was determined using a luciferase assay system (Promega) and measured with a Perkin Elmer Victor^{3}V multilabel counter. Where 5 μCi of [^{35}S]Met (MP Biomedical) (>1,000 Ci/mmol) was used to label the translation products, the translation reaction mixtures contained 10 μM each amino acid except methionine. Translation reactions were stopped by adding sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) loading buffer (Invitrogen) for a final 1× concentration. [^{35}S]Met-labeled translation products were analyzed on SDS-PAGE gels and visualized using a GE Typhoon Trio phosphorimager.

RESULTS

Sertraline is potently fungicidal against various *Cryptococcus* isolates *in vitro*. In our previous screen of the Johns Hopkins Clinical Compound Library composed of 1,514 drugs approved by the FDA or by one or more foreign governmental agencies (40), we found that sertraline exhibited a modest inhibitory effect on *Aspergillus nidulans*. We investigated the activity of this antidepressant against *Cryptococcus*, given the brain involvement of cryptococcal infections and the psychotropic property of this drug. Sertraline displayed high potency against H99, a clinical and reference *Cryptococcus* strain. The MIC_{90} against H99 was approximately 6 μg/ml (Table 1), which is much lower than the reported MICs against many other fungal strains of *Candida* or *Aspergillus* species (12, 13).

To determine whether sertraline is fungistatic or fungicidal, we performed a comparative time course assay of cell viability in the presence of sertraline and the commonly used fungistatic drug fluconazole. We cultured *Cryptococcus* H99 cells in RPMI medium or in PBS buffer to represent rapidly proliferative or quiescent cells, respectively. These cells were treated with no drug, sertraline alone, fluconazole alone, or a combination of sertraline and fluconazole. At different time points, aliquots of cell suspensions were spread onto drug-free solid medium to determine the number of viable cells by measuring the CFU. In RPMI medium, the number of viable cryptococcal cells started to decrease at as early as 2 h in the presence of sertraline compared to the no-drug control; there were no viable fungal cells in the presence of sertraline

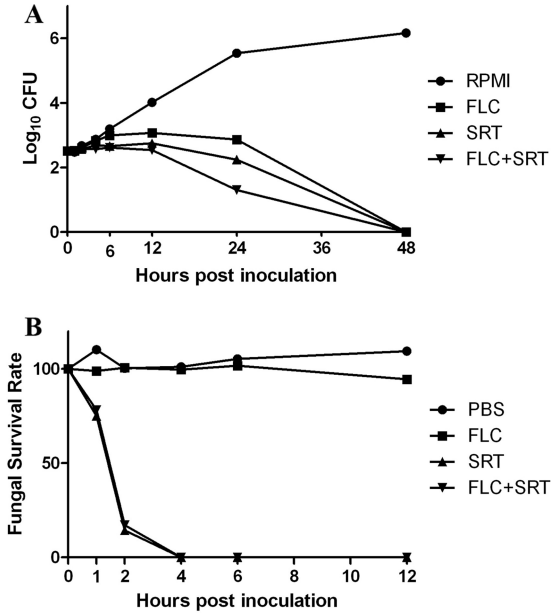

FIG 1 Sertraline is fungicidal against both proliferative and quiescent *Cryptococcus* cells. (A) H99 cells were inoculated into RPMI media and cultured without any drug (control) or in the presence of fluconazole (FLC; 8 μg/ml) or sertraline (SRT; 10 μg/ml) or a combination of these two drugs. At the indicated time points, aliquots of cell suspensions were transferred and plated onto drug-free agar medium to determine CFU after 2 more days of incubation. Fungal cells proliferated rapidly in the absence of any drugs, but they were gradually cleared with any of the drug treatments. (B) H99 cells were inoculated into PBS buffer and cultured without any drug (control) or in the presence of fluconazole (8 μg/ml) or sertraline (8 μg/ml) or a combination of these two drugs. At the indicated time points, aliquots of cell suspensions were transferred and plated on drug-free medium to determine CFU after 2 more days of incubation. Fungal cells in PBS maintained viability during the tested period.

after 48 h of incubation (Fig. 1A). Sertraline was modestly more effective than fluconazole under these conditions (Fig. 1A). The drug combination showed the highest efficiency in clearing the yeast cells (Fig. 1A). Similarly, H99 cells in PBS buffer were rapidly cleared by sertraline (within 4 h after inoculation), indicating that sertraline is fungicidal and is potent against *Cryptococcus* cells regardless of whether cells are growing or not (Fig. 1B). In sharp contrast, fluconazole showed no effect on the viability of the fungal cells in PBS buffer (Fig. 1B). The lack of killing effect of fluconazole on quiescent fungal cells is consistent with the classification of fluconazole as a fungistatic drug: that is, it is effective in inhibiting fungal cell proliferation but ineffective in destroying cells that are not actively growing. The fungicidal effect of sertraline is not specific to *Cryptococcus*, as sertraline also killed *S. cerevisiae* BY4742 cells regardless of whether they were growing in RPMI medium or quiescent in PBS buffer (see Fig. S1 in the supplemental material). These results indicate that sertraline is able to kill fungal cells independently of whether they are proliferating.

To examine whether the susceptibility to sertraline is specific to the H99 strain or is a general trait of *Cryptococcus*, we further tested 23 genetically and phenotypically distinct *Cryptococcus* strains, including 13 serotype A (this serotype accounts for 95% of clinical cryptococcosis cases), 6 serotype D (serotype D represents fewer than 5% of clinical cases of cryptococcosis), 3 serotype A-serotype D hybrids, and 1 *C. gattii* strain (Table 1). These strains are clinical or environmental isolates from North America, Europe, or Africa. The MICs of sertraline were determined by the microdilution assay according to the CLSI standard. Compared to the wide range of inhibitory concentrations of fluconazole (from 1 to >64 μg/ml), all of these strains are very sensitive to sertraline, with a surprisingly narrow spectrum of inhibitory concentrations (≤10 μg/ml) (Table 1). Notably, there is no correlation between the levels of susceptibility to fluconazole and to sertraline among these strains, indicating that there is no cross-resistance between sertraline and fluconazole. Thus, it is unlikely that these two drugs share common mechanisms of action. These results imply the general susceptibility of *Cryptococcus* strains to sertraline irrespective of their level of susceptibility to fluconazole.

Sertraline interacts synergistically or additively with fluconazole against *Cryptococcus in vitro*. As fluconazole is the most commonly used antifungal in cryptococcosis treatment, drugs that can act additively or synergistically with fluconazole are more likely to be used clinically. In the time course assay described above, we observed that the combination of sertraline and fluconazole accelerated the clearance of proliferative *Cryptococcus* cells (Fig. 1A). To determine the interaction between sertraline and fluconazole against *Cryptococcus*, we compared the susceptibility of the 24 isolates to a combination of these drugs with their susceptibility to each drug alone using the disk diffusion halo assay. For all the strains tested, halos formed near disks containing the drug combination were larger and clearer than those formed near disks containing either fluconazole or sertraline alone (data not shown, but see Fig. S2 in the supplemental material). The results indicate possible synergistic or additive interactions between sertraline and fluconazole against *Cryptococcus*.

We next quantified the interaction between sertraline and fluconazole against *Cryptococcus* by using a microdilution assay to determine the fractional fungicidal concentration index (FFCI). Sertraline increases the antifungal activity of fluconazole against all the tested *Cryptococcus* strains, demonstrating a synergistic (FFCI < 0.5) or additive (FFCI between 0.5 and 1.0) (4) effect of the combination of the two drugs (Table 1). This is consistent with the results obtained by the disk diffusion method. Considering the divergence of the genetic backgrounds of the tested strains, these observations suggest the possibility that the combination of sertraline and fluconazole could be more effective at clearing different *Cryptococcus* isolates in cases of mammalian cryptococcosis.

Sertraline alone or in combination with fluconazole displays antifungal activity in a murine model of systemic cryptococcosis. As described earlier, sertraline exerts anti-*Cryptococcus* activity alone or in combination with fluconazole *in vitro*. To determine the efficacy of sertraline or its combination with fluconazole *in vivo*, we adopted a murine model of systemic cryptococcosis. We chose the intravenous model of cryptococcosis here, because our pilot experiments showed that organ fungal burden displayed relatively low variations among individual animals. To examine the drug efficacy in this model, we assigned mice into four groups: the control (PBS) group, the fluconazole treatment group, the sertraline treatment group, and the group receiving a drug combination. Given that it takes at least 5 to 7 days for sertraline to reach a steady state in the mouse brain (8), mice of the sertraline group and the drug combination group were given a 7-day pretreatment of sertraline prior to the infection by the *Cryptococcus* H99 reference strain. Based on previous studies, the serum level of sertraline in these animals was likely to be too low (55 to 250 ng/ml [34]) to

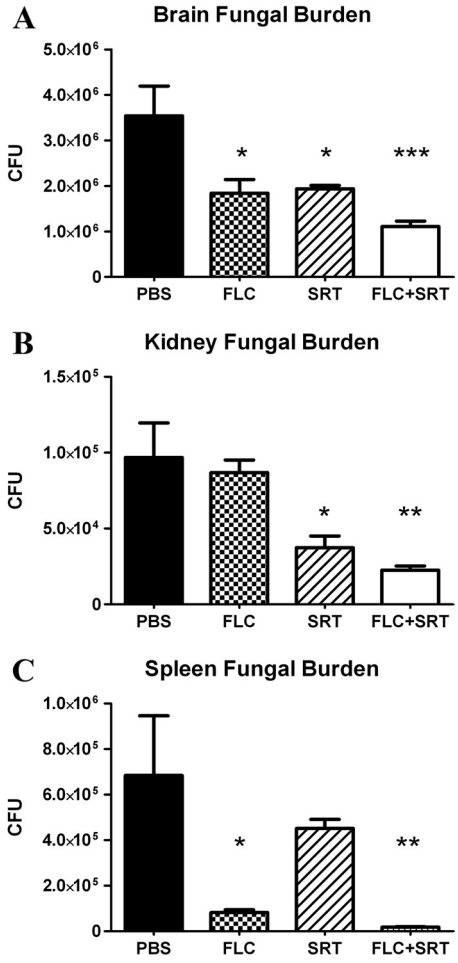

FIG 2 Sertraline reduces the fungal burden alone or in combination with fluconazole *in vivo*. Brains (A), kidneys (B) and spleens (C) of mice from different treatment groups were dissected and homogenized. The suspensions were diluted serially, and the fungal burden was determined by calculating CFU. Sertraline alone significantly reduced the brain and the kidney fungal burden, while the drug combination reduced the fungal burden significantly in all three organs. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. In brain, the drug combination displayed higher potency compared to either fluconazole or sertraline alone (both $P < 0.05$). The efficacy of the drug combination is also superior to that of fluconazole alone in kidney ($P < 0.001$) or that of sertraline alone in spleen ($P < 0.001$).

exert any growth-inhibitory effect on the fungal cells initially inoculated into the bloodstream. Fluconazole treatment was initiated at 1 day postinfection (dpi 1) in both the treatment group administered fluconazole alone and the drug combination treatment group. Such treatment was continued for 3 consecutive days. The animals were sacrificed at dpi 4; their brain, kidney, and spleen tissues were homogenized and the fungal burdens of these organs were determined by measuring CFU. As shown in Fig. 2, the treatment with sertraline alone reduced the fungal burden in the brain ($P < 0.05$), with its efficacy comparable with that of fluconazole. Sertraline also reduced fungal burden in kidney, while fluconazole showed no apparent effect. Sertraline appeared to have a modest effect on fungal burden in spleen, but such effect was not statistically significant. The differences in the levels of drug efficacy for the three organs examined may reflect variations of drug concentrations at these organs. Importantly, the treatment with the combination of sertraline and fluconazole displayed the most potent efficacy in reducing fungal burden of all three organs examined (Fig. 2). Therefore, the data demonstrate that sertraline alone is efficacious against cryptococcosis and that the combination of sertraline with fluconazole is a more effective treatment than either drug alone due to their strong synergy *in vivo*.

Sertraline antagonizes the growth-inhibitory effect of fluconazole on many *Candida* strains. To examine if the potency of sertraline alone or in combination with fluconazole can be extended to *Candida* species, we tested the MICs of sertraline against six strains that represent six different *Candida* spp.: *C. albicans* SC5314, *C. glabrata* PAT2ISO3, *C. krusei* DUMC132.91, *C. parapsilosis* MMRL1594, *C. tropicalis* MMRL2017, and *C. lusitaniae* 2-367. The MIC_{90}s ranged from 12 to 24 μg/ml (Table 2), which are considerably higher than the MICs against *Cryptococcus* but are consistent with previous studies (13, 37). These MIC values are higher than the achievable blood or even organ levels of sertraline.

In striking contrast to the results obtained with *Cryptococcus*, sertraline had a significant antagonistic impact on the inhibitory effect of fluconazole for the majority of the tested *Candida* stains *in vitro* (Fig. 3). For example, 1-day incubation with fluconazole alone was able to effectively inhibit the growth of the *C. tropicalis* MMRL2017 strain at a concentration of 1.0 μg/ml. However, with the addition of sertraline (~1.0 to 6.0 μg/ml), fungal growth in the presence of fluconazole not only recovered but actually became more robust. The spectrum of the concentrations at which sertraline exerts an antagonistic effect with fluconazole unfortunately falls in the clinically relevant range. Similar antagonistic interactions between sertraline and fluconazole were also observed among three other *Candida* strains: *C. albicans* SC5314, *C. glabrata* PAT2ISO3, and *C. parapsilosis* MMRL1594 (Fig. 3). Sertraline did not decrease the inhibitory effect of fluconazole on *C. lusitaniae* 2-367 or on *C. krusei* DUMC132 (Fig. 3). Although synergistic or additive interactions between sertraline and fluconazole were reported in studies of some *Candida* strains (30), antagonistic interactions between fluconazole and sertraline are commonly observed among *Candida* and *Aspergillus* strains (this study and reference 7).

Sertraline interferes with translation in fungal cells. The broad antiproliferation activity of sertraline against many organisms indicates that the mechanism underlying its fungicidal activity may involve some fundamental cellular processes. To identify the potential fungal molecules and processes affected by sertraline, we screened the whole-genome deletion collection of *S. cerevisiae* for sertraline-sensitive or sertraline-resistant mutants. This mutant collection set is composed of a nonessential gene deletion set in a haploid background and an essential gene deletion in a heterozygous diploid background (35). After one round of initial

TABLE 2 Sertraline is less potent against *Candida* strains

Strain	MIC_{90} (μg/ml)	MIC_{100}[a] (μg/ml)
Candida albicans SC5314	32	48
Candida glabrata PAT2ISO3	12	16
Candida krusei DUMC132.91	12	16
Candida parapsilosis MMRL1594	24	24
Candida tropicalis MMRL2017	24	32
Candida lusitaniae 2-367	8	24

[a] MIC_{100}, the lowest drug concentration that resulted in a 100% decrease in absorbance.

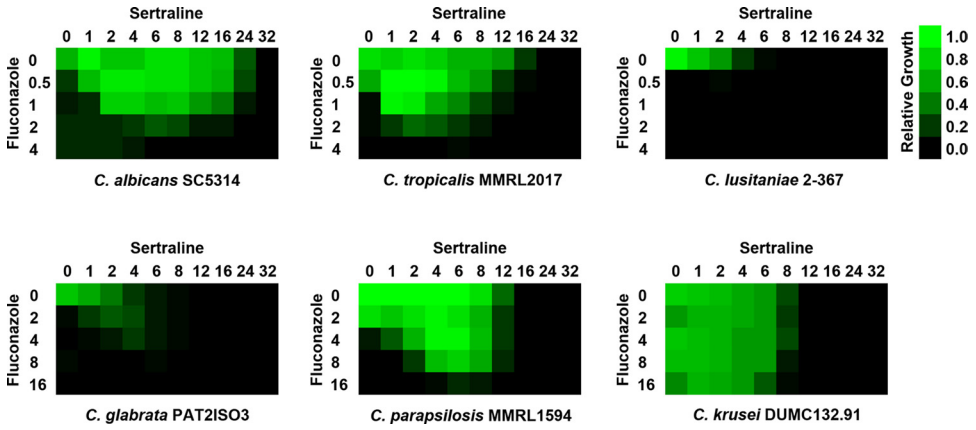

FIG 3 Antagonistic effects between sertraline and fluconazole among *Candida* strains. Six *Candida* strains, *C. albicans* SC5314, *C. glabrata* PAT2ISO3, *C. krusei* DUMC132.91, *C. parapsilosis* MMRL1594, *C. tropicalis* MMRL2017, and *C. lusitaniae* 2-367, were incubated in RPMI media with the indicated drug treatment. Gradients in columns represent sertraline (shown in micrograms per milliliter); gradients in rows represent fluconazole (shown in micrograms per milliliter). The scale of the fluconazole concentrations was determined based on a previous study (40). Growth was measured by absorbance at 600 nm after 24 h of incubation. Green indicates fungal growth, and black indicates the lack of fungal growth.

screening and two rounds of additional screening to confirm the selected phenotype, 88 resistant and 36 sensitive strains were identified (see Table S2 in the supplemental material). Gene ontology analyses indicated that these genes are enriched for those with roles in intracellular vesicle transport and membrane organization (Fig. 4), which is consistent with the findings of two recent studies of the effects of sertraline on yeast (27, 30).

Interestingly, genes related to protein synthesis are highly enriched in the resistant group; the most sensitive mutant selected from our screen was strain Δ*tif3*, in which an important translation initiation factor Tif3 was disrupted. Since previous studies in mammalian cells have indicated that sertraline inhibits translation initiation (15), these data indicated that it was possible that sertraline also disrupts translation in fungi.

Sertraline inhibits translation in a *Cryptococcus* cell-free system. To determine the effect of sertraline on translation in *Cryptococcus*, we performed *in vitro* translation assays. In these assays, the luciferase mRNA was used as the template and the translation machinery was provided by the cell extract obtained from *C. neoformans* strain H99. As expected, translation in these *Cryptococcus* cell extracts was synergistically dependent on the mRNA 5′ terminal cap and 3′ terminal poly(A) tail, as with *Saccharomyces* cell extract (Fig. 5A) and *Neurospora crassa* cell extract (33, 36). This result verified that the *Cryptococcus in vitro* translation system faithfully recapitulated the dependence of cap and poly(A) for translation.

The effects of sertraline on translation of cap and poly(A) luciferase mRNA were assessed by measurement of both (i) the enzymatic activity of the luciferase produced by the *in vitro* translation and (ii) the level of [^{35}S] methionine incorporation into the luciferase polypeptide produced. We found that sertraline inhibited the translation efficiency in a dose-dependent manner using both detection methods (Fig. 5B and C). Luciferase enzyme activity dropped 50% compared to the control when the concentration of sertraline was increased to 0.1 mM (30.6 μg/ml). No enzyme activity above the background was detected in the presence of sertraline at 0.4 mM (122.4 μg/ml). The decrease in luciferase enzyme activity in the presence of sertraline was not due to direct interference of sertraline with the enzymatic activity of the synthesized luciferase, since, based on this measurement, addition of sertraline into the cell extract after translation was completed did not alter enzyme activity (Fig. 5B). The [^{35}S] methionine incorporation assay showed that sertraline affected the yield of luciferase polypeptide synthesized in this cell extract (Fig. 5C). Protein synthesis was also affected by sertraline in fungal extracts derived from the yeast *S. cerevisiae* and the filamentous fungus *N. crassa*, although higher concentrations of sertraline were required to achieve a similar level of inhibition (see Fig. S3 in the supplemen-

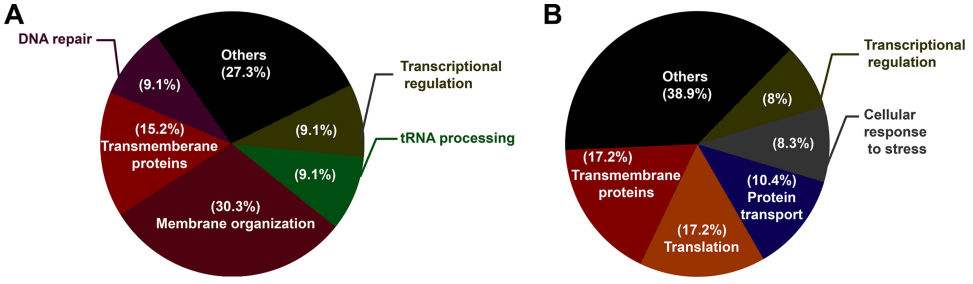

FIG 4 Gene ontology analysis of the *S. cerevisiae* genes involved in sertraline tolerance or susceptibility. Gene ontology (GO) terms for annotated *S. cerevisiae* genes involved in sertraline resistance (A) or susceptibility (B) were extracted from the GO database and sorted into the immediate subcategories for molecular functions and biological processes. See Table S2 in the supplemental material for the detailed gene list.

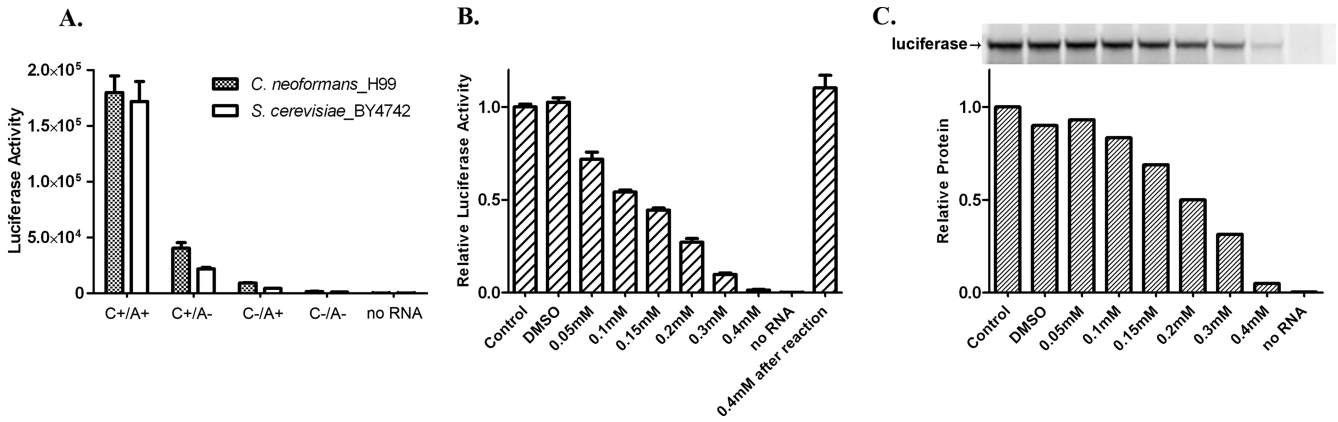

FIG 5 Sertraline inhibits translation in a *Cryptococcus* cell-free system. The cell-free translation system was prepared as described in Materials and Methods. (A) The translation of luciferase in both *Cryptococcus* and *Saccharomyces* cell extracts is dependent on the mRNA 5′ terminal cap, designated C, and on the 3′ terminal poly(A) tail, designated A. The cap and poly(A) synergistically stimulate RNA translation in both cell-free systems. (B and C) Water, the solvent DMSO, or sertraline in stock solution was added into reaction mixtures to reach the indicated concentrations. Luciferase protein synthesis by the cell-free translation system was measured based on the relative light units arising from the enzymatic activity of luciferase (B) or the level of [^{35}S]methionine incorporated into the synthesized luciferase polypeptide (C). Both measurements showed that sertraline inhibited translation in a dose-dependent manner.

tal material). In contrast, fluconazole, which is known to target the enzyme Erg11 in the ergosterol biosynthetic pathway, did not show any inhibitory effect on protein synthesis in a similar concentration range in such assays (see Fig. S4 in the supplemental material). These data obtained in cell-free translation systems using fungal extracts show that sertraline interferes with fungal protein synthesis.

DISCUSSION

Sertraline offers a promising option for the treatment of cryptococcosis, especially cryptococcal meningitis. Here we provide evidence for the potent anticryptococcal activity of the antidepressant sertraline both *in vitro* and *in vivo*. Given the difficulties in developing antifungal drugs *de novo*, recent studies have explored existing clinical compounds for potential use as antifungals (39). Existing pharmaceutical and safety information concerning the use of these drugs in animals or in humans could greatly accelerate the investigation into their clinical use as antifungals. Consistent with the safety profile of sertraline for long-term use in patients, we did not observe any severe side effects due to sertraline administration during the treatment process in the animal studies presented here. Previous studies also showed the safety of sertraline administration in mice with a similar daily dose but for a much longer period of time (25).

The discovery that sertraline has anticryptococcal activity offers the potential for an additional choice for treating cryptococcosis. Our tests of 24 diverse *Cryptococcus* strains indicate a uniformly high sensitivity of this fungus to sertraline relative to other fungal species tested. Compared to fluconazole, sertraline showed a much narrower range of inhibitory concentrations against these diverse *Cryptococcus* isolates (Table 1), suggesting a lower probability of naturally occurring resistance to sertraline in existing *Cryptococcus* populations. The fungicidal nature of sertraline and its synergy with fluconazole against *Cryptococcus*, as previously observed *in vitro* (21) and *in vivo* in an insect model (30) and in this study shown in a mammalian model, could potentially shorten the duration of anticryptococcal therapy and reduce the risk of emerging drug resistance. During latent infection or during fluconazole treatment, *Cryptococcus* cells are likely dormant or grow slowly. Given that even dormant cells need transcription and translation (22), and that sertraline is capable of killing fungal cells under quiescent conditions, it is reasonable to speculate that sertraline might be useful to clear latent cryptococcal infections or to kill residual fungal cells unharmed by the fluconazole treatment. This could be tested by further investigation.

It is worth mentioning that we did not observe any differences in survival in any of the treatment groups (including the fluconazole-treated groups). This is most likely due to the rapid disease progression in this model. The A/J mouse used in this study is very susceptible to *Cryptococcus* infections, and H99 is one of the most virulent clinical isolates of *Cryptococcus* (38). We tried different inocula of the fungal cells (1×10^3, 1×10^4, and 1×10^5 cells/mouse) in this intravenous infection model and found that a 10-fold decrease in the inoculum prolonged survival for an additional 1 to 2 days and that infected mice all succumbed to the diseases within 8 days. We speculate that better protection against cryptococcosis by sertraline or the drug combination might be observed in other animal models or in humans, or if cryptococcosis is caused by less virulent strains. Optimization of drug doses, the route and frequency of drug administration, and the duration of treatment warrants further investigation in order to assess the treatment outcomes.

One of the most valuable aspects of sertraline as a potential anticryptococcal drug is its superior ability to accumulate in the CNS relative to other antifungals. This is particularly critical in the treatment of cryptococcosis, given that *Cryptococcus* preferentially proliferates in the brain. Consistent with our expectations, the *in vivo* study presented here supports the hypothesis of the ability of sertraline, either alone or in combination with fluconazole, to reduce brain fungal burden.

Although sertraline demonstrates efficacy against cryptococcosis comparable to that of fluconazole based on the data presented here, the potential application of this drug to treat mycoses caused by fungal pathogens such as *Candida* or *Aspergillus* requires further investigation. The commonly observed antagonistic interaction between sertraline and fluconazole against *Candida*

and *Aspergillus* strains is particularly concerning (this study and reference 7). It is possible that specific chemical modification of sertraline could increase its efficacy against these other fungi and abolish its antagonistic interaction with fluconazole. Such modifications would increase its value in the battle against systemic mycoses.

The antifungal mechanisms of sertraline. Sertraline displays extremely broad antiproliferation activity against evolutionarily diverse organisms (10, 12–15, 19, 20, 23). Recent studies indicate the influence of sertraline on membrane stability or vesicle transport in fungi (27, 30). The results of our *S. cerevisiae* mutant screens are consistent with these discoveries. Our mutant screens and the *in vitro* translation assays also indicated protein synthesis as another process interfered with by sertraline. Our finding regarding the inhibitory effect of sertraline on translation is consistent with a recent study on translation in tumor cells (15), even though the implicated mammalian factors in the mTOR pathway identified in that study, such as PDCD4 or REDD1, do not have obvious homologs in fungi. Inhibition of translation could possibly cause changes in other processes, as molecules involved in protein trafficking and membrane proteins were also significantly enriched in our screen (Fig. 4; see also Table S2 in the supplemental material). Sertraline's impact on translation might more acutely affect protein synthesis from specific transcripts in *Cryptococcus* important for growth such that it does not need to completely inhibit protein synthesis for its strong anticryptococcal activity. Thus, while sertraline inhibits mammalian protein synthesis, it may be that qualitative, not quantitative, differences in sertraline's effects on fungal protein synthesis are crucial for sertraline's antiproliferation activity.

The possibility of emergence of fungal resistance to sertraline seems low based on the following observations. First, we noted during our genetic screen that *S. cerevisiae* gene deletion mutants selected for sertraline resistance showed an increase in MIC of only up to 50% compared to the wild type. Second, our repeated attempts to perform UV mutagenesis with *S. cerevisiae* and *C. neoformans* failed to yield any resistant strains with sertraline MICs greater than 14 μg/ml. Third, all natural *Cryptococcus* strains tested showed uniformly high sensitivity to sertraline. Such observations are drastically different from what is known for azole drugs, as a more than 10- to 100-fold difference in fluconazole susceptibilities can be easily observed in both clinical and laboratory settings. This feature, although making it rather challenging to pinpoint the underlying fungicidal mechanisms of sertraline, could be advantageous for its clinical application, as the possibility of encountering sertraline-resistant *Cryptococcus* isolates and the risk of developing fungal resistance during therapy would be low.

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